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Amino Acid Sequence of Sardine Calcitonin and Its Hypocalcemic Activity in Rats

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ABSTRACT—A novel calcitonin (CT) was isolated from the spotlined sardine, *Sardinops melanostictus*. The primary structure of sardine CT was determined as follows: H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Ala-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Ser-Tyr-Pro-Arg-Thr-Asn-Val-Gly-Ala-Gly-Thr-Pro-NH_2. This amino acid sequence was different from that of salmon CT in 4 amino acid residues at positions 8th, 21th, 27th and 29th. As judged by the international method of CT bioassay, hypocalcemic activity of sardine CT was calculated as 4156 IU/mg. When compared for durability of CTs, it was found that sardine CT was significantly more potent than that of salmon CT. This is the first report of CT from a marine species of teleost.

INTRODUCTION

Calcitonin (CT) is a hormone, composed of 32 amino acid residues [9]. The primary structures of CTs from 3 species of teleosts (salmon [4], eel [5] and goldfish [6]), have been sequenced to date. However, CT from teleost which inhabits the sea throughout its life, has not yet been isolated. Marine teleosts are always exposed to high ambient Ca in which its level is 2–3 times higher than serum Ca levels. On the other hand, it is known that in mammals, bile is an important pathway for Ca excretion [12], and that CT stimulates excretion of Ca into the bile [11]. It is reported that also in marine teleosts, bile Ca concentrations are 3–7 times higher than serum Ca levels, which are higher than those of fresh water fish [2, 7]. Therefore, if teleosts CTs play a similar role in Ca regulation of bile as in mammals, CT might be more important in marine teleosts. In fact, it is reported that administration of salmon CT to a marine teleost (kelp bass) produced significant hypocalcemia, although there are many conflicting results regarding the effect of CT when it was administered to fresh water fishes [1]. In the present study, the primary structure of sardine CT was studied as one of the representatives of CTs from marine teleosts. Hypocalcemic activity of sardine CT was also examined by the rat bioassay.

MATERIALS AND METHODS

Purification of sardine CT

Three hundred twenty individuals of spotlined sardine (*Sardinops melanostictus*) were provided by fishermen in May 1993 in Toyama Bay. Pharyngeal tissue including UBG was dissected out, and immediately frozen until use. Sardine CT was purified according to the method reported for ray CT [8].

The crude extract of sardine UBG was subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on an ODS-120T column (4.6×250 mm, Tosoh) with a linear gradient elution from 20 to 80% CH_3CN in 0.1% trifluoroacetic acid (TFA) for 60 min. In each fraction eluted, the presence of CT-specific immunoactivity was examined using Western blotting method with salmon CT polyclonal antiserum. The immunopositive fraction was further purified on the same column with a linear gradient from 40 to 80% CH_3OH in 0.1% TFA for 50 min.

The purified sardine CT was subjected to a protein sequencer (Model 473A, Applied Biosystems). After determining the primary structure, sardine CT was synthesized with a peptide synthesizer (Model 430A, Applied Biosystems). Furthermore, retention time of the synthetic sardine CT in RP-HPLC was compared with that of natural sardine CT.

Rat bioassay

Hypocalcemic activity of synthetic sardine CT was examined using synthetic one and compared with that of synthetic salmon CT by using rat bioassay according to Uchiyama *et al.* [10]. Each rat received 1 pM of sardine CT or 1 pM of salmon CT, contained in 400 μl of vehicle solution (0.9% saline solution containing 0.1% bovine serum albumin, pH 4.6). The same volume of vehicle solution was also administered to rat as a control. Blood was sampled before (zero hr) and at 0.5, 1, 2 and 3 hr after administration of CT or vehicle.

Serum Ca and Na concentrations were determined with atomic absorption spectrophotometer (180–70 type, Hitachi-Zeeman). Furthermore, the area between the serum Ca curve and the initial (zero) level was taken as a measure of the hormone response or control during the 3 hr period after administration, as reported previously [6]. In this warp, the duration of the hypocalcemic potency of the hormone could be expressed quantitatively. Data were analyzed by Student's *t*-test.

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RESULTS

Purification and amino acid sequence of sardine CT

At first, an aliquot of 1/10 volume of crude extraction was subjected to RP-HPLC (Fig. 1). Location of the peak of sardine CT was sought on the result of Western blotting (shown by the arrow in Fig. 1). The consequence obtained in the second subjecting with a different solvent system of RP-HPLC are also exhibited in Figure 2. Then, the purification of sardine CT is judged from the single peak obtained. A half amount of eventual sample was subjected to a peptide sequencer to determine the amino acid sequence of sardine CT which is as follows: H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Ala-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Ser-Tyr-Pro-Arg-Thr-Asn-Val-Gly-Ala-Gly-Thr-Pro-NH_2

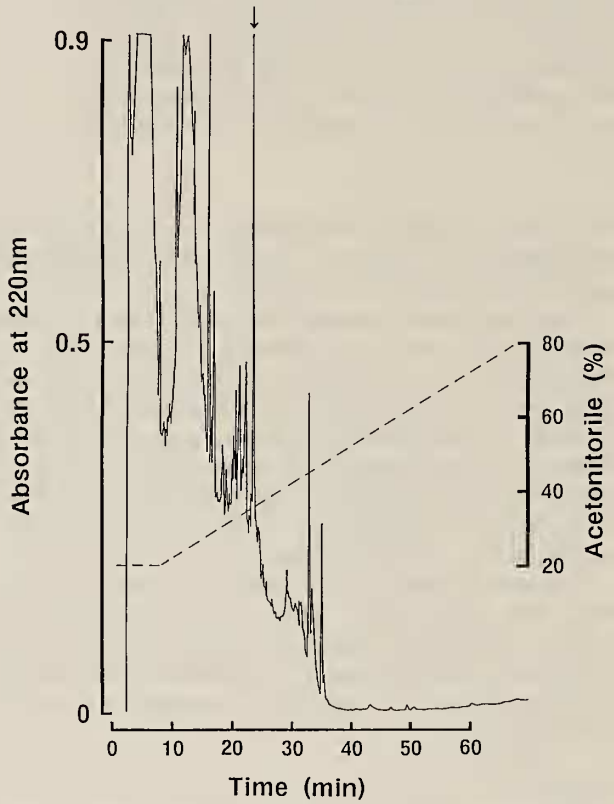

FIG. 1. Reverse phase HPLC on an ODS-120T column. Sample: crude extract of ultimobranchial glands of sardine; flow rate: 1 ml/min; Solvent system: linear gradient elution from 20 to 80% CH_3CN in 0.1% trifluoroacetic acid for 60 min. Arrow indicates the peak containing sardine calcitonin.

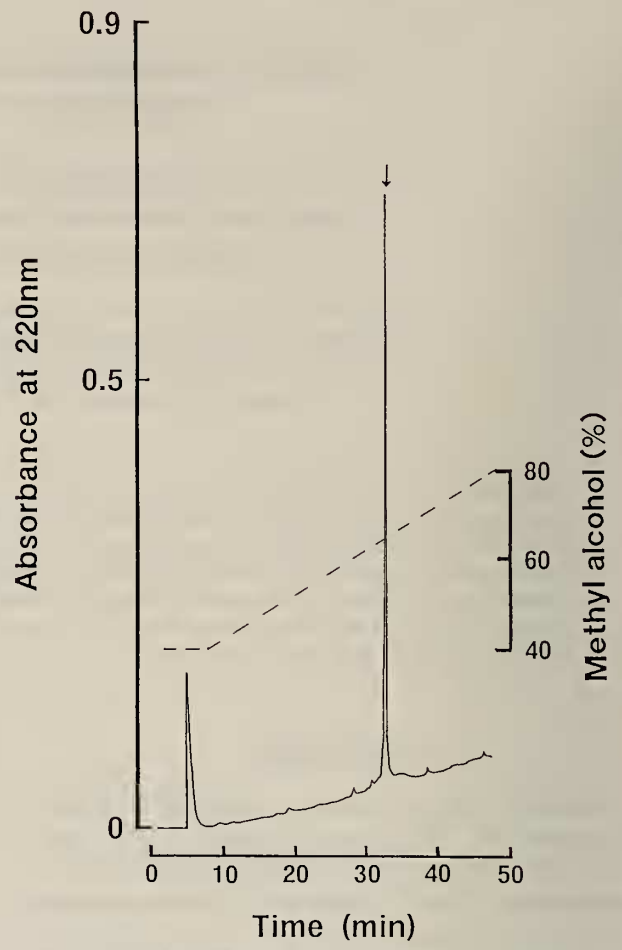

FIG. 2. Reverse phase HPLC on an ODS-120T column. Sample: the peak containing sardine calcitonin; flow rate: 1 ml/min; Solvent system: linear gradient elution from 40 to 80% CH_3OH in 0.1% trifluoroacetic acid for 50 min. Arrow indicates the peak of purified sardine calcitonin.

(Fig. 3). Furthermore, it was known that retention time of the synthesized CT in RP-HPLC was coincident with that of natural sardine CT.

Hypocalcemic potency of sardine CT by rat bioassay

Administration of 1 pM of sardine CT evoked significant hypocalcemia at 0.5 hr ($P<0.001$), 1 hr ($P<0.001$) and 2 hr after ($P<0.05$). (Fig. 4). On the other hand, administration of salmon CT produced smaller declines in serum Ca levels than these obtained for sardine CT (Fig. 4). Hypocalcemic

	1									10									20									30			
sardine CT	C	S	N	L	S	T	C	A	L	G	K	L	S	Q	E	L	H	K	L	Q	S	Y	P	R	T	N	V	G	A	G	T	P-NH_2
salmon CT	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	N	T	G	S	G	T	P-NH_2
eel CT	C	S	N	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	D	V	G	A	G	T	P-NH_2
fowl CT	C	A	S	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	D	V	G	A	G	T	P-NH_2
goldfish CT	C	S	S	L	S	T	C	V	L	G	K	L	S	Q	E	L	H	K	L	Q	T	Y	P	R	T	N	V	G	A	G	T	P-NH_2

FIG. 3. Amino acid sequences of sardine calcitonin and other calcitonins. The identical amino acid residues in salmom lineage are boxed. Amino acid residues of sardine CT, which are identical to salmon calcitonin are underlined.

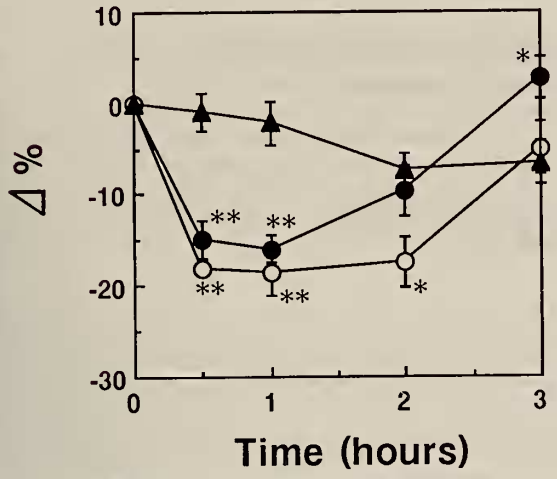

FIG. 4. Changes in serum Ca levels after administrations of either 1 pmol of sardine calcitonin (○), 1 pmol of salmon calcitonin (●) or vehicle (▲). Vertical bars indicate ± SE. The number of rats used were 10 individuals for sardine CT, 10 individuals for salmon CT and 6 individuals for vehicle. * and ** indicate significant differences from vehicle-$P<0.05$ and $P<0.001$, respectively.

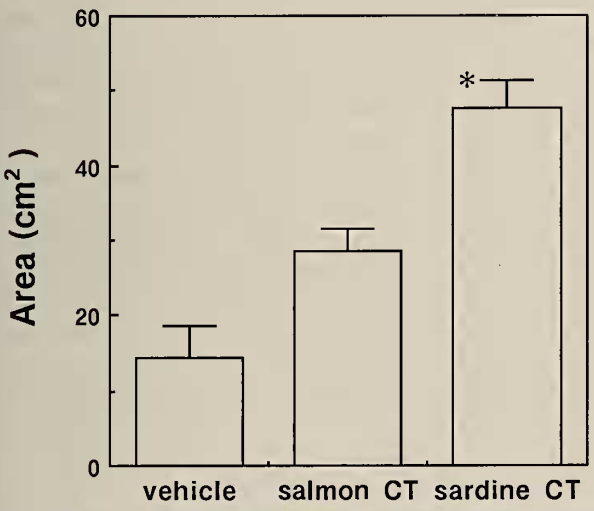

FIG. 5. Total areas below the initial level of serum Ca after administrations of either 1 pmol of sardine calcitonin or salmon calcitonin during 3 hour. Vertical bars indicate ± SE. * indicates significant difference from salmon CT-$P<0.001$

activity evoked after administration of salmon CT was recovered by 2 hr. The areas below the initial levels are shown in Figure 5. The area of the nonspecific declines caused by the vehicle solution was 14.4 ± 4.1 cm^2. The respective values of sardine CT and salmon CT were 47.7 ± 3.8 cm^2 and 28.4 ± 3.0 cm^2. The area of the former was significantly larger than that of the latter ($P<0.001$).

Serum Na levels did not change during 3 hr after administration, and remained at about 330 mg/100 ml (data not shown).

DISCUSSION

Eleven kinds of CTs have been sequenced to date. These are classified into 3 lineages: human lineage (human, rat and rabbit), porcine lineage (porcine, cattle, sheep and dog) and salmon lineage (salmon, eel, fowl and goldfish) [6]. Recently, we purified stingray CT from a cartilaginous fish [8], which appears to belong to another lineage. The primary structure of sardine CT sequenced in the present study demonstrates that it should be placed with the salmon lineage. In composition of amino acids, the sardine CT differs from eel CT (at positions 8th, 21th and 26th; homology 91%), from goldfish CT (at positions 3th, 8th and 21th; homology 91%), from salmon CT (at positions 8th, 21th, 27th and 29th; homology 88%), and from fowl CT (at positions 2th, 3th, 8th, 21th and 26th positions; homology 84%). Thus, it seems that among the salmon lineage, the sardine CT is more similar to eel and goldfish CTs in term of amino acid compositions. These results also imply that there is no distinction in amino acid components of CTs between fresh water teleosts and marine teleosts. The primary structure of CT appears to be well conserved among teleosts. Homology between sardine CT and mammalian CTs is low, 47–50% in human lineage and 38–47% in porcine lineage. However, similarity of sardine CT to ray CT is relatively high (69%).

As judged from declines in serum Ca levels of rat at 1 hr after administration, the activity of sardine CT was calculated as 4,156 IU/mg, as against a value of salmon CT which is reported as 3,500 IU/mg [3]. Furthermore, the present study demonstrates that sardine CT is more effective than salmon CT as its effect is of larger potency and long lasting.

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REFERENCES

1 Glowacki J, O'sullivan J, Miller M, Wilkie DW, Deftos LJ (1985) Calcitonin produces hypocalcemia in leopard sharks. Endocrinology 116: 827–829

2 Hickman C, Trump BE (1969) In "Fish physiology Vol 1" Ed by WS Hoar and DJ Randall, Academic Press, New York and London, pp 91–239

3 Homma T, Watanabe M, Hirose S, Kanai A, Kangawa K, Matsuo H (1980) Isolation and determination of the amino acid sequence of chicken calcitonin I from chicken ultimobranchial glands. J Biochem 100: 459–467

4 Niall HD, Keutmann HT, Copp DH, Potts JT Jr (1969) Amino acid sequence of salmon ultimobranchial calcitonin. Proc Natl Acad Sci USA 64: 771–778

5 Otani M, Yamaguchi H, Megro T, Kitazawa S, Watanabe S, Orimo H (1976) Isolation and characterization of calcitonin from pericardium and esophagus of eel. J Biochem 79: 345–352

6 Sasayama Y, Ukawa K, Kai-ya H, Oguro C, Takei Y, Watanabe TX, Nakajima K, Sakakibara S (1993) Goldfish calcitonin:

purification, characterization and hypocalcemic potency. Gen Comp Endocrinol 89: 189–194

7 Suzuki N, Sasayama Y (1992) Changes of mineral concentrations of body fluids in some seawater fishes transferred to diluted seawater. Proc Jap Soc Comp Endocrinol **1992**: 41

8 Takei Y, Takahashi A, Watanabe TX, Nakajima K, Sakakibara S, Sasayama Y, Suzuki N, Oguro C (1991) New calcitonin isolated from the ray, *Dasyatis akajei*. Biol Bull 180: 485–488

9 Reginster JY (1993) Calcitonin for prevention and treatment of osteoporosis. Am J Med 95 (Suppl 5A): 44–47

10 Uchiyama M, Yoshihara M, Murakami T, Oguro C (1978) Presence of a hypocalcemic factor in the ultimobranchial gland of snake. Gen Comp Endcrinol 36: 59–62

11 Yamaguchi M, Yamamoto T (1979) Effects of various calcitonins on calcium concentrations in the bile and serum of thyroparathyroidectomized rats. Chem Pharm Bull 27: 1671–1674

12 Yamaguchi M, Yamamoto T, Hasegawa A (1979) Physiological significance of calcium excretion into the bile of rats. Chem Pharm Bull 27: 3137–3139